α-Tocopherol preserves endothelial cell migration in the presence of cell-oxidized low-density lipoprotein by inhibiting changes in cell membrane fluidity

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Objective: **Endothelial cell (EC) migration is essential for healing areas of arterial injury and angioplasty sites. Iron or copper-oxidized low-density lipoprotein ($oxLDL_{Cu}$) inhibits EC migration in vitro, but the effect of physiologically relevant monocyte/macrophage-oxidized LDL ($oxLDL_{cell}$) is unknown. We postulated that $oxLDL_{cell}$ would inhibit EC migration and that this inhibition would be reversed by antioxidants.**
Methods: **The effect of $oxLDL_{Cu}$ and $oxLDL_{cell}$ on EC migration was studied by using a razor scrape assay, and migration was assessed after 24 hours. In addition, ECs were incubated with various antioxidants, including butylated hydroxytoluene (BHT), probucol, or α-tocopherol, for 1 hour prior to initiation of the scrape assay and application of oxLDL.**
Results: **Both $oxLDL_{Cu}$ and $oxLDL_{cell}$ inhibited migration. The antioxidants did not alter the antimigratory activity of $oxLDL_{Cu}$, but α-tocopherol preserved EC migration in the presence of $oxLDL_{cell}$. The lack of effect of BHT or probucol suggested that the effect of α-tocopherol resided not in its antioxidant activity but in its membrane-stabilizing properties. To test this theory, the effect of oxLDL and α-tocopherol on relative cell membrane fluidity was assessed by fluorescence recovery after photobleaching. Both $oxLDL_{Cu}$ and $oxLDL_{cell}$ increased relative membrane fluidity. Preincubation with α-tocopherol inhibited the increase in membrane fluidity of ECs incubated in $oxLDL_{cell}$ but not in $oxLDL_{Cu}$.**
Conclusions: **These studies show that α-tocopherol preserves EC migration in $oxLDL_{cell}$ and hastens restoration of the endothelial monolayer after injury by inhibiting changes in membrane integrity caused by oxLDL. (J Vasc Surg 2004; 39:229-37.)**

Clinical Relevance: **Recent studies find that vitamin E is not efficacious in the secondary prevention of cardiovascular events, perhaps because vitamin E does not efficiently block oxidation pathways known to be operative in atherosclerotic arteries. "Non-antioxidant" properties of vitamin E, however, could be important in the primary prevention of atherosclerosis and its complications. Our in vitro studies show that α-tocopherol can preserve endothelial migration in the presence of cell-oxidized LDL. This effect might improve the healing of endothelial injuries at sites of arterial repair or angioplasties, especially in lipid-laden arterial walls.**

Endothelial cell (EC) migration is central to many normal and pathologic processes, including wound healing, tumor angiogenesis, and restoration of endothelial integrity after arterial injury. EC migration in vitro is stimulated by growth factors and inhibited by oxidized low-density lipoprotein (oxLDL) produced by incubation with iron or copper ions.[1,2] Oxidized lipids accumulate in the atherosclerotic arterial wall and might impair EC migration in vivo. Delayed re-endothelialization of an arterial injury or angioplasty site could allow prolonged smooth muscle cell (SMC) proliferation, resulting in restenosis. In fact, oxLDL has been implicated as contributing to restenosis after coronary angioplasty.[3] We have shown that products of lipid oxidation accumulate in prosthetic grafts in vivo,[4] but the effect on EC migration onto the graft is not known.

Most in vitro studies use oxLDL generated by incubating LDL with micromolar concentrations of transition metal ions, such as iron or copper ions ($oxLDL_{Cu}$). Because unbound, transition metal ions are not likely to exist in plasma, where most copper and iron ions are bound to ceruloplasmin and transferrin, respectively, the applicability of in vitro EC migration studies to in vivo endothelial repair is unclear. ECs, SMCs, and monocytes can oxidize LDL, and cell-modified LDL ($oxLDL_{cell}$) is more physiologically relevant than $oxLDL_{Cu}$. Differences in the biochemistry of cell-mediated and copper-mediated oxLDL have been identified.[5] Compared with $oxLDL_{Cu}$, $oxLDL_{cell}$ is characterized by a slower rate of formation, lower levels of thiobarbituric acid–reacting substances (TBARSs), limited inhibition by superoxide dismutase, and the requirement of nanomolar (rather than micromolar) concentrations of transition metal ions during the oxidation process.[5]

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$OxLDL_{cell}$ has many biologic effects that are similar to $oxLDL_{Cu}$, but the effect of $oxLDL_{cell}$ on EC migration has not been reported previously. As an initial step in studying the relevance of inhibition of EC migration by oxLDL, we tested the ability of LDL oxidized by activated monocytes to inhibit EC migration. $OxLDL_{cell}$ inhibited EC migration to a similar degree as $oxLDL_{Cu}$.

Because studies in hypercholesterolemic animals have suggested that α-tocopherol attenuates intimal hyperplasia and restenosis after balloon injury,[6,7] we investigated the effectiveness of α-tocopherol and other chain-breaking antioxidants in preserving EC migration in vitro. Antioxidants were ineffective in preventing the inhibition of EC migration by $oxLDL_{Cu}$, but α-tocopherol did preserve migration of ECs incubated in $oxLDL_{cell}$. Investigation into the mechanism of action suggested that the ability of α-tocopherol to maintain EC membrane fluidity in the normal range was essential for the preservation of migration in oxLDL.

METHODS

Preparation and modification of lipoproteins. LDL (d = 1.019-1.063) was isolated by sequential ultracentrifugation from freshly drawn, citrated normolipemic human plasma to which EDTA was added prior to ultracentrifugation.[8] The purity was assessed by gel electrophoresis, and all preparations were assayed for protein, total cholesterol (Boehringer Mannheim Corp, Indianapolis, Ind), endotoxin (Pierce Chemical Co, Rockford, Ill), and TBARSs by using the method of Schuh et al.[9] TBARSs, one measure of lipid oxidation, were expressed as nanomoles malondialdehyde (MDA) per milligram cholesterol. Immediately prior to use, LDL was dialyzed against saline at 4°C for 48 hours to remove the EDTA. Three different methods of oxidizing LDL were used, two using activated monocytic (U937) cells and one using $CuSO_4$.

To prepare cell-modified LDL, Dacron- or zymosan-activated U937 cells (American Type Culture Collection, Rockville, Md) were used. Dacron graft material used to activate U937 cells was prepared as previously described.[10] Briefly, Cooley Double Velour Knitted Dacron Graft (Meadox Medicals, Inc, Oakland, NJ) was cut into pieces measuring 1.25 × 1.25 cm and weighing 80 ± 5 mg; sequentially washed in 10 mmol/L EDTA, buffer, ethanol, and deionized water; and then plasma-sterilized. LDL oxidized by Dacron-activated U937 cells ($oxLDL_{Dac}$) was prepared by combining a Dacron graft piece, LDL (1000 μg), and U937 cells (500,000) in 1 mL RPMI (that contains no added transition metal ions) and incubating for 24 hours, then centrifuging to remove graft fibers and cellular debris. The supernatant was analyzed for cholesterol recovery and TBARSs. Cell-modified LDL using zymosan (from *Saccharomyces cerevisiae*; Sigma Chemical Co, St. Louis, Mo) to activate the U937 cells ($oxLDL_{zym}$) was prepared similarly, replacing graft material with 4 mg zymosan prepared as described by Ehrenwald et al.[11]

$OxLDL_{Cu}$ was prepared by incubating 1 mL LDL (5-8 mg cholesterol/mL) with 1 μmol/L $CuSO_4$ for 16 to 24 hours at 37°C until the clear yellow solution became colorless. Oxidation was terminated by addition of 5 μmol/L EDTA. $OxLDL_{Cu}$ was analyzed for TBARSs prior to use.

EC culture and migration assay. ECs from adult bovine aortas were isolated by gentle scraping after collagenase exposure and subcultured in Dulbecco modified Eagle medium and Ham F12 Nutrient Mixture (DMEM/F12, 1:1, vol/vol) containing 5% fetal bovine serum (FBS). ECs were used between passages 5 and 17.

EC migration was measured using a "razor scrape" assay as previously described.[1,12] ECs were grown to confluence in 12-well tissue culture plates, made quiescent by incubation in serum-free DMEM containing 0.1% gelatin for 24 hours before use. To begin the migration assay, a razor blade was pressed into the plastic to mark the starting line, and cells on one side of the mark were removed with the blade. Cells were washed, and colorless RPMI 1640 medium with 0.1% gelatin and test compound was added. Migration was terminated at 24 hours by fixing with modified Wright-Giemsa stain. A 256-gray level, 640 × 480 pixel image of cells was captured with a digital charged-coupled device camera mounted on a phase-contrast microscope. Migration was measured by a semi-automated computer-assisted procedure in which the number of cells that crossed the starting line was determined in two randomly selected fields, each 1500 μm wide, in every well. All experiments were performed in triplicate with at least two different LDL preparations and two different cell isolates. Results were expressed as the mean number of migrating cells per 3000 μm of starting line ± SEM.

Protein synthesis measurement. Cell-associated protein synthesis was determined by measuring [^{3}H]leucine incorporation into trichloroacetic acid (TCA)-precipitable material as previously described.[13] Briefly, ECs were incubated with [^{3}H]leucine for 24 hours, lysed, and protein precipitated with chilled 5% TCA. The precipitate was solubilized, and radioactivity was determined in a scintillation counter and expressed as the percentage of applied [^{3}H]leucine incorporated into TCA-precipitable material × 1000.

Fluorescence recovery after photobleaching. ECs were seeded at a density of 40,000/cm^2 in DMEM/F12 with 5% FBS, allowed to adhere for 24 hours, made quiescent in serum-free DMEM/F12 for 18 hours, and then incubated in RPMI 1640 medium with 0.1% gelatin. After 3 hours of treatment with agonist, the ECs were washed, labeled with 1-acyl-2-(N-4-nitrobenzo-2-oxa-1,3 diazole)-amino-caproyl phosphatidylcholine (a fluorescent phospholipid analogue; Molecular Probes, Eugene, Ore) during a 20-minute incubation at 37°C, and then tested for fluorescence recovery after photobleaching (FRAP).[14] A small area of a cell (1-2 μm in diameter) was photobleached with a 75-millisecond pulse of an argon laser (488 nm, 100% power) through a 40× long working distance objective of an ACAS-570 interactive laser cytometer (Meridian Instruments, Inc, Okemos, Mich). Recovery of fluorescence within the bleached area, because of lateral diffusion of neighboring unbleached fluorophore, was assessed by

repetitive scanning across the cell surface with an attenuated laser beam (30% power) at 488 nm for 90 seconds. Rate and extent of recovery reflected relative membrane microviscosity. Twenty to thirty individual measurements were made for each test condition. Mean values with SEM were calculated. ECs from three different isolates, and LDL from three separate preparations were used to verify findings.

Statistics. All data represent the mean ± SEM. Experiments were performed in triplicate with at least three different cell isolates. Data evaluation was performed by *t* test or analysis of variance by using GraphPad InStat (GraphPad Software, Inc, San Diego, Calif). Differences were considered statistically significant at $P < .05$.

RESULTS

Effect of $oxLDL_{Dac}$ on EC migration. The effect of $OxLDL_{Dac}$ and $OxLDL_{Cu}$ on EC migration was compared. Both $OxLDL_{Dac}$ and $oxLDL_{Cu}$ (TBARS, ranging from 3 to 7 nmol MDA/mg cholesterol) caused nearly complete inhibition of EC migration that was statistically significant (Fig 1, *A-B*). Native LDL, which was not significantly oxidized (TBARS, ranging from 0.2 to 0.7 nmol MDA/mg cholesterol), did not inhibit EC migration and, in fact, stimulated migration. To determine whether other factors from the graft material, or secreted by graft-activated U937 cells, inhibited EC migration, medium incubated with Dacron graft material and LDL, or medium incubated with Dacron graft material and U937 cells, was also tested. In no case was EC migration inhibited (Fig 1, *A-B*), suggesting that the LDL modified by Dacron-activated monocytes was responsible for the inhibition of EC migration.

To assure that inhibition of EC migration by $oxLDL_{Dac}$ and $oxLDL_{Cu}$ was not due to cytotoxicity or death, protein synthesis and reversibility of the inhibition of EC migration were examined. No significant change in protein synthesis was noted between ECs treated with medium alone, native LDL, $oxLDL_{Dac}$, or $oxLDL_{Cu}$ (data not shown), suggesting that cell injury and/or death was not responsible for the decrease in movement. Reversibility of the inhibition was demonstrated by resumption of EC movement following removal of $oxLDL_{Dac}$ or $oxLDL_{Cu}$ 24 hours after initiation of the migration assay (Fig 1, *C*). Migration during the second 24-hour period, with no $oxLDL_{Dac}$ or $oxLDL_{Cu}$ present, was not significantly different from the movement of untreated ECs during a 24-hour period, indicating that inhibition of migration by both forms of oxidized LDL was reversible.

Antioxidant protection of EC migration. The oxidative stress caused by oxLDL is postulated to be responsible for inhibition of migration. If this is the mechanism, antioxidants should restore migration. To assess this theory, ECs were preincubated for 1 hour in α-tocopherol (vitamin E), probucol, or butylated hydroxytoluene (BHT), which have been reported to protect ECs from oxidative stress.[15-17] EC migration in the presence of $oxLDL_{Dac}$ or $oxLDL_{Cu}$ and the antioxidant was measured after 24 hours. Vitamin E consistently preserved EC migration in $oxLDL_{Dac}$, but neither probucol nor BHT was protective (Fig 2, *A-B*). None of the tested antioxidants preserved migration in $oxLDL_{Cu}$ (Fig 2, *C-D*), despite

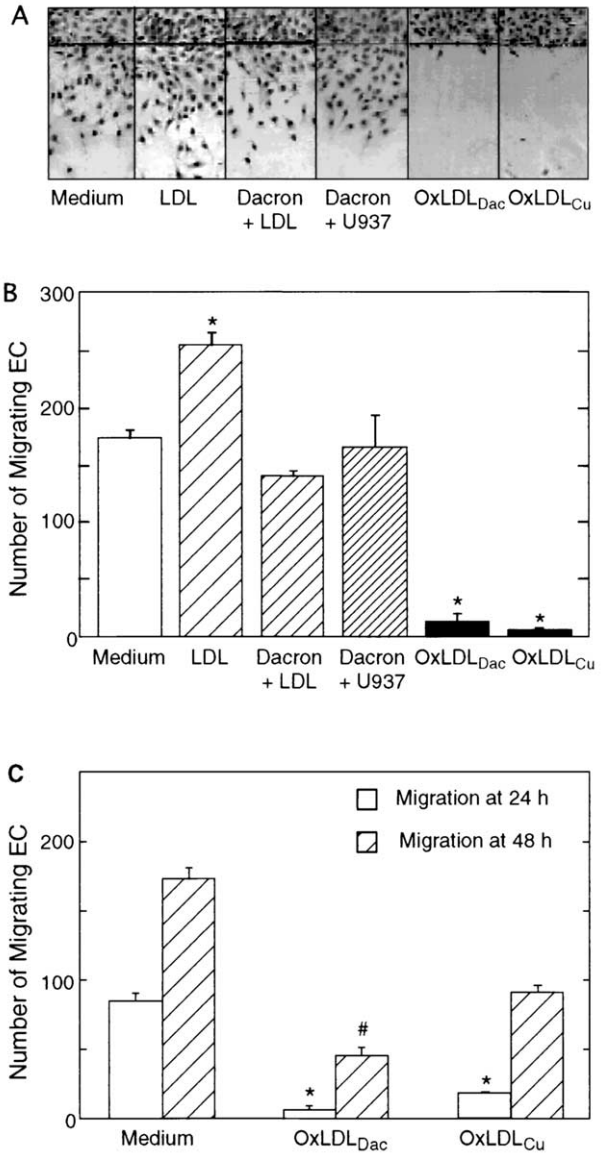

Fig 1. Inhibition of EC migration by $oxLDL_{Dac}$. EC migration assay was performed with native LDL (400 μg cholesterol/mL; TBARS, 0.4), LDL incubated with Dacron graft material (400 μg cholesterol/mL; TBARS, 1.1), conditioned medium from U937 cells incubated with Dacron graft material, $oxLDL_{Dac}$ (400 μg cholesterol/mL; TBARS, 3.7), or $oxLDL_{Cu}$ (400 μg cholesterol/mL; TBARS, 3.8). Results are shown in pictorial form (**A**) and graphic form (**B**). The reversibility of the inhibition of migration was assessed by removing oxLDL after 24 hours, replacing medium with RPMI 1640 with 0.1% gelatin, allowing migration to proceed for an additional 24 hours, and then measuring EC movement by image analysis (**C**). *$P < .001$ versus EC migration in medium. #$P < .05$ versus EC migration at 24 hours in medium.

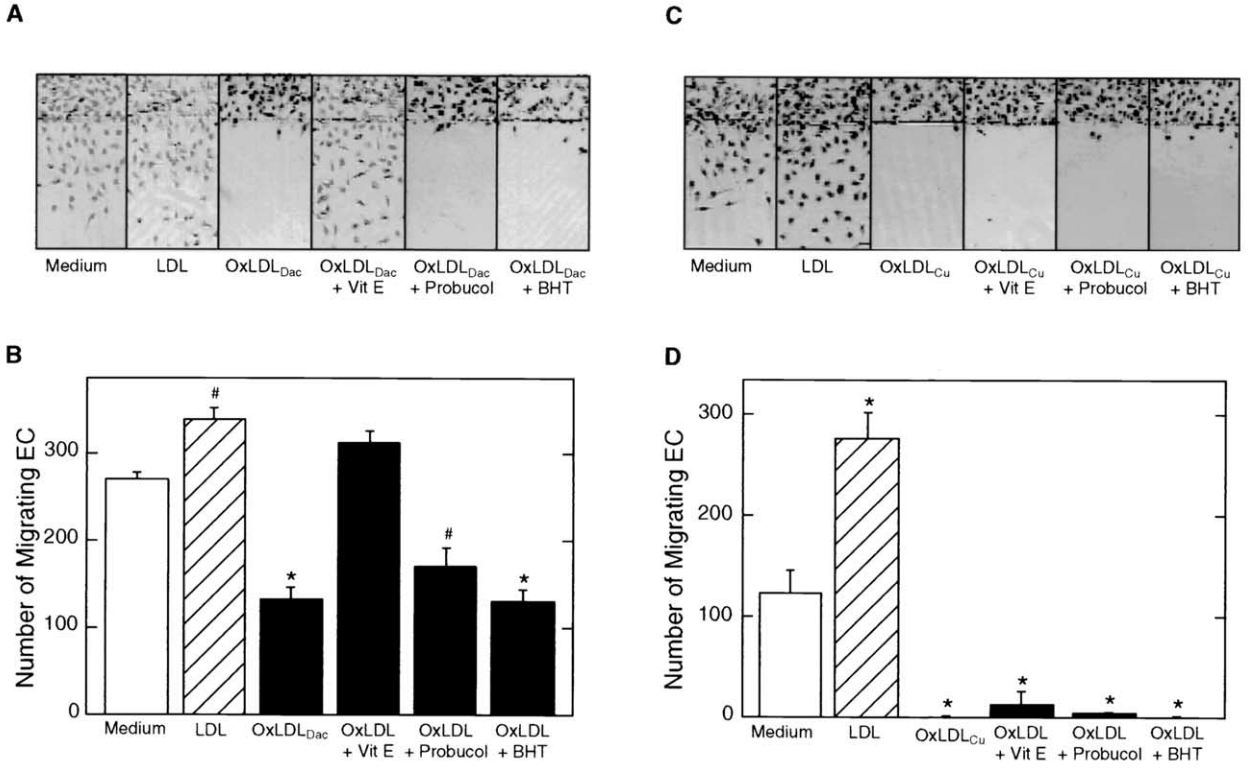

Fig 2. Vitamin E preserves EC migration in oxLDL$_{Dac}$. Confluent, quiescent ECs were preincubated with 50 μmol/L vitamin E, 40 μmol/L probucol, or 10 μmol/L BHT for 1 hour. Migration was then initiated in the presence of antioxidant and 400 μg/mL oxLDL$_{Dac}$ (TBARS, 8.0) or oxLDL$_{Cu}$ (TBARS, 6.6). Medium alone or native LDL (TBARS, 0.7) served as control. The number of ECs migrating across the starting line at 24 hours was quantitated. Results with oxLDL$_{Dac}$ are shown in pictorial form (**A**) and graphic form (**B**). Results with oxLDL$_{Cu}$ are shown in pictorial form (**C**) and graphic form (**D**). *$P \leq .001$ versus EC migration in medium. #$P < .05$ versus EC migration in medium.

similar degrees of oxidation for oxLDL$_{Dac}$ and oxLDL$_{Cu}$, TBARSs of 8.0 and 6.6 nmol MDA/mg cholesterol, respectively. α-Tocopherol (50 μmol/L) was capable of preserving EC migration over a wide range of concentrations of oxLDL$_{Dac}$ (Fig 3, *A*). Increasing concentrations of α-tocopherol up to 200 μmol/L were incapable of protecting EC migration in the presence of oxLDL$_{Cu}$ (Fig 3, *B*). Furthermore, the use of EDTA and $CuSO_4$ to oxidize the LDL did not account for α-tocopherol's inability to preserve migration in oxLDL$_{Cu}$, because the addition of similar quantities of EDTA and $CuSO_4$ to oxLDL$_{cell}$ did not alter the preservation of EC migration by α-tocopherol (data not shown). Another antioxidant vitamin, ascorbic acid, did not preserve EC migration in oxLDL and did not augment the effect of α-tocopherol (data not shown). These data suggested that the antioxidant properties of α-tocopherol did not account for its ability to preserve EC migration in oxLDL$_{Dac}$.

Effect of zymosan-activated U937 cell-modified LDL on EC migration. To determine whether other forms of oxLDL$_{cell}$ had properties similar to oxLDL$_{Dac}$, the effect of oxLDL$_{zym}$ on EC migration was studied. OxLDL$_{zym}$ significantly inhibited EC migration (Fig 4, *A*), whereas supernatant collected from preparations of zymosan incubated with medium alone, with U937 cells alone, or with LDL alone did not inhibit EC migration. This finding confirmed that oxLDL, not compounds within the zymosan preparation or secreted by the cells in response to zymosan, was responsible for inhibition of EC migration. The inhibition of migration was reversible with removal of the oxLDL$_{zym}$ (Fig 4, *B*), as previously noted with oxLDL$_{Dac}$ and oxLDL$_{Cu}$. α-Tocopherol preincubation consistently preserved EC migration in oxLDL$_{zym}$ but not oxLDL$_{Cu}$ (Fig 4, *C*). These results suggested that oxLDL$_{cell}$, generated by Dacron- or zymosan-activated monocytic cells, had properties distinct from oxLDL$_{Cu}$.

Preloading with α-tocopherol preserves migration. The requirement for α-tocopherol's presence in the medium during the migration assay was assessed. ECs were preloaded with α-tocopherol for 1 hour prior to initiation of the migration assay. In only half of the wells was α-tocopherol replaced after the razor scrape and addition of oxLDL$_{Dac}$. Migration was preserved with equal efficacy when α-tocopherol was preloaded only or when it was also

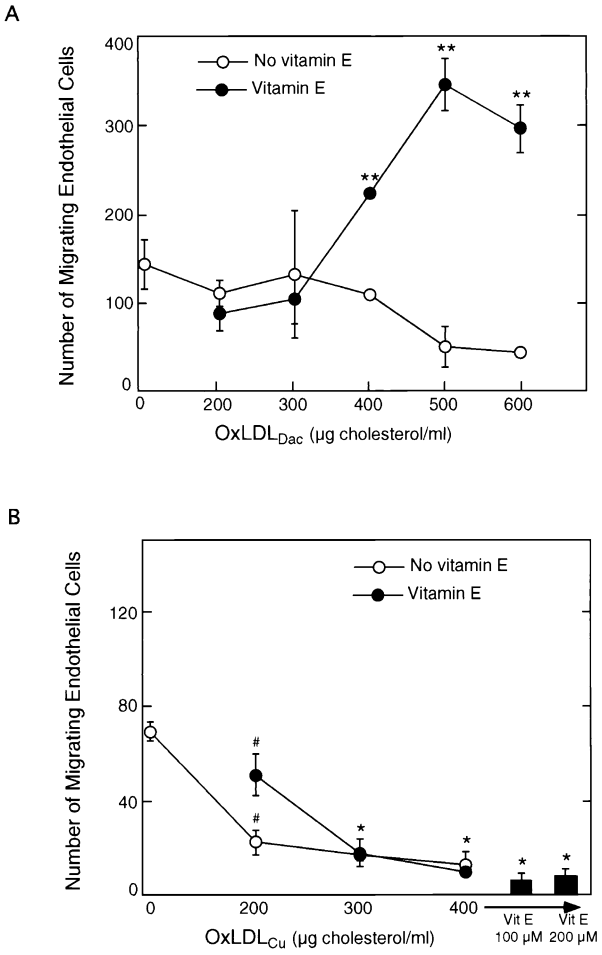

Fig 3. Effectiveness of vitamin E over a range of oxLDL concentrations. Confluent, quiescent bovine aortic ECs were preincubated in 50 μmol/L vitamin E for 1 hour, and then migration was initiated in the presence of vitamin E and varying concentrations of oxLDL$_{Dac}$ (TBARS, 4.5; **A**)or oxLDL$_{Cu}$ (TBARS, 4.4; **B**). At maximal inhibition of EC migration by oxLDL$_{Cu}$, higher concentrations of vitamin E were also examined. *$P < .001$ versus EC migration in medium. #$P < .05$ versus EC migration in medium. **$P < .001$ versus EC migration in same concentration of oxLDL without vitamin E.

present during the 24-hour migration assay. This finding suggested that the effect of vitamin E was on the cell rather than on the lipoprotein in the medium.

Membrane fluidity changes. In addition to antioxidant properties, α-tocopherol has membrane-stabilizing effects that result from incorporation of α-tocopherol into cell membranes. Therefore, the changes in the relative membrane fluidity of ECs because of oxLDL$_{Dac}$, oxLDL$_{zym}$, and oxLDL$_{Cu}$, in the presence and absence of α-tocopherol, was studied. All three forms of oxidized LDL significantly increased relative membrane fluidity when compared with ECs in medium alone (Fig 5). These results were confirmed with anisotropy measurements (data not shown). When ECs were preincubated with α-tocopherol prior to addition of the various forms of oxLDL, changes in relative membrane fluidity were prevented only in ECs exposed to oxLDL$_{Dac}$ and oxLDL$_{zym}$, the two cell-modified LDLs. ECs incubated in α-tocopherol prior to exposure to oxLDL$_{Dac}$ or oxLDL$_{zym}$ had no statistically significant difference in FRAP measurements compared with ECs incubated in medium alone. However, relative membrane fluidity was increased by oxLDL$_{Cu}$ even in ECs preincubated with α-tocopherol. When α-tocopherol and native LDL alone were added to ECs, membrane fluidity decreased slightly from control levels, consistent with previous findings.[18]

DISCUSSION

OxLDL has been the focus of many investigations because of its potential role in the initiation and progression of atherosclerotic lesions. In vitro studies of oxLDL's effect on cellular function have used LDL modified by a variety of methods, most commonly LDL oxidized with transition metal ions such as copper or iron. Although oxLDL$_{cell}$ is more relevant to conditions in vivo, few investigations have compared the effect of oxLDL$_{Cu}$ and oxLDL$_{cell}$ on specific cellular functions. Our studies demonstrate for the first time that oxLDL$_{cell}$ inhibits EC migration and are consistent with previous reports that iron- and copper-oxidized LDLs inhibit EC migration.[1,2] The more surprising finding is that α-tocopherol preserves migration of ECs incubated in oxLDL$_{cell}$ but not in oxLDL$_{Cu}$. The inability of α-tocopherol to restore migration in the presence of oxLDL$_{Cu}$ is consistent with a previous report,[2] but the finding that α-tocopherol preserves migration in the presence of oxLDL$_{cell}$ is novel.

Antioxidants retard the formation of oxLDL and the development of neointimal hyperplasia after balloon injury in animal models, but their effects on cell function is not fully defined. The antioxidants used in this study, BHT, probucol, and α-tocopherol, have been shown to protect ECs from oxidative stress.[15-17] BHT and probucol are synthetic, whereas α-tocopherol is the major lipid-soluble antioxidant present in human plasma. α-Tocopherol can act as a peroxyl radical scavenger and break the chain reaction associated with lipid peroxidation. BHT, another chain-breaking antioxidant, does not preserve migration, suggesting that other characteristics of α-tocopherol are responsible for its protective effect. α-Tocopherol intercalates into cell membranes, and its location in the EC membrane might contribute to its effectiveness. Probucol, however, has also been shown to be incorporated into the EC membrane where it can act as a radical scavenger,[16] so simple intramembrane location of the antioxidant is unlikely to account for the effectiveness of α-tocopherol.

Membrane microviscosity is a critical determinant of cell migration. Ghosh et al[18] show a biphasic migratory response to changes in membrane microviscosity. A moderate decrease in membrane fluidity increases EC migration, but greater decreases inhibit migration. However, increases in membrane fluidity also inhibit migration.

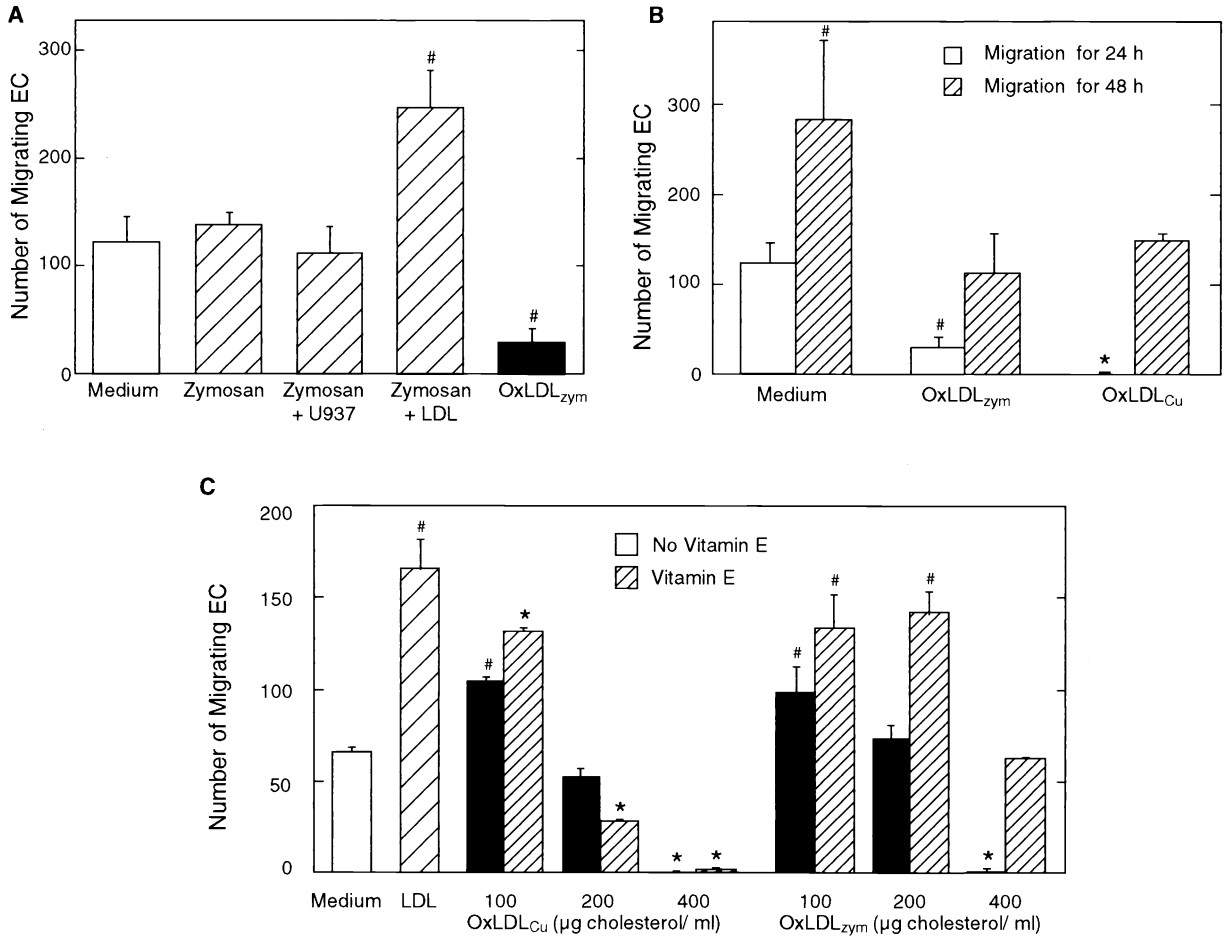

Fig 4. OxLDL$_{zym}$ inhibits EC migration. EC migration assay was performed with RPMI medium containing 0.1% gelatin, medium exposed to zymosan for 24 hours, conditioned medium from U937 cells incubated with zymosan for 24 hours, LDL incubated with zymosan (400 µg cholesterol/mL; TBARS, 0.9), or LDL incubated with zymosan and U937 cells resulting in oxLDL$_{zym}$ (400 µg cholesterol/mL; TBARS, 6.7). EC migration was quantitated by image analysis at 24 hours (**A**). The reversibility of the inhibition by oxLDL$_{zym}$ was assessed in a parallel set of wells by removing oxLDL$_{zym}$ after 24 hours, adding fresh medium without oxLDL, and quantitating migration after an additional 24 hours. Migration during the 24 hours after removal of oxLDL was not significantly different than 24-hour migration in medium alone (**B**). To determine the effectiveness of vitamin E in preserving EC migration in the presence of oxLDL$_{zym}$, cells were preincubated in vitamin E (50 µmol/L) for 1 hour. Migration was initiated, and varying concentrations of oxLDL$_{zym}$ or oxLDL$_{Cu}$ (TBARS, 3.0) were added with vitamin E (50 µmol/L). In control wells, no vitamin E was added before or after initiation of the razor scrape assay. Migration was assessed after 24 hours (**C**). *$P \leq .001$ versus EC migration in medium. #$P \leq .05$ versus EC migration in medium.

Changes in membrane microviscosity are known to influence the function of membrane-bound proteins. Increased membrane fluidity markedly decreases membrane-bound Rac, a member of the Rho-GTPase (guanosine triphosphatase) family that regulates polymerization of actin in the protrusion of lamellipodia necessary for migration.[18] Altered physical properties of the membrane can also directly influence cytoskeletal function and lamellipodial extension.[19] The present study shows that both oxLDL$_{Cu}$ and oxLDL$_{cell}$ increase membrane fluidity and inhibit EC migration.

The ability of α-tocopherol to preserve EC migration in oxLDL$_{cell}$ could be the result of optimization of membrane fluidity. Vitamin E intercalates into cell membranes, decreases membrane fluidity, and abrogates lysophosphatidylcholine (lysoPC)-induced changes in microviscosity.[18] However, probucol, despite being incorporated into the cell membrane, has not been shown to possess any EC membrane-stabilizing activity, as a result of steric and charge considerations. This finding could account for the difference in effectiveness of vitamin E and probucol in preserving EC migration.

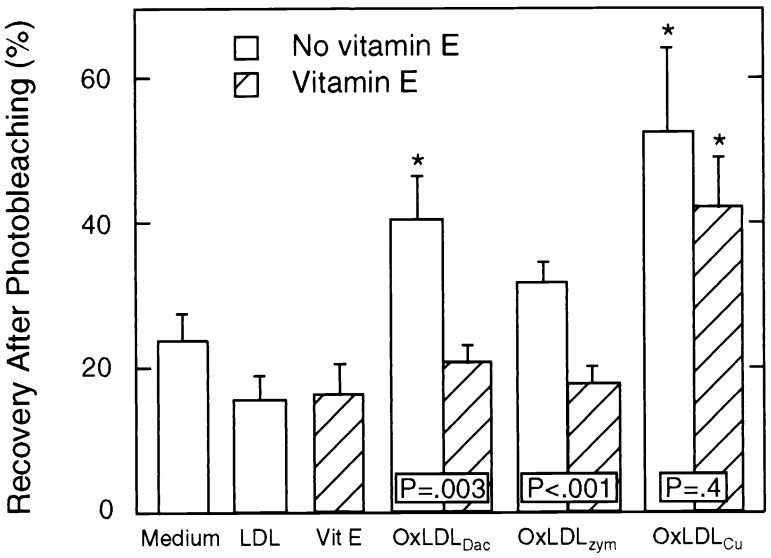

Fig 5. Effect of oxLDL$_{cell}$ and oxLDL$_{Cu}$ on membrane fluidity. ECs were incubated with native LDL (TBARS, 0.2), oxLDL$_{Dac}$ (TBARS, 6.6), oxLDL$_{zym}$ (TBARS, 6.3), or oxLDL$_{Cu}$ (TBARS, 6.3) for 3 hours. In parallel wells, ECs were preincubated with vitamin E (50 μmol/L) for 1 hour prior to the addition of oxLDL$_{Dac}$, oxLDL$_{zym}$, or oxLDL$_{Cu}$ for 3 hours. The fluorescent phospholipid analogue was added, and then fluorescence recovery was measured after laser photobleaching. $*P \leq .05$ versus recovery by ECs in medium. The statistical difference between recovery in oxLDL with or without vitamin E preincubation is shown in the boxed insets.

Interestingly, preincubation with α-tocopherol abrogates the increase in EC membrane fluidity in oxLDL$_{cell}$ but not in oxLDL$_{Cu}$, and this finding parallels the preservation of migration. The similarity of TBARS of the oxLDL$_{Cu}$ and oxLDL$_{cell}$ and the concentration range of α-tocopherol used suggest that neither LDL oxidation level nor insufficient vitamin E explains the differences observed. Furthermore, ECs are enriched with α-tocopherol prior to incubation with oxLDL, so equivalent amounts of α-tocopherol would be incorporated into the cell membranes. Boissonneault et al[2] suggest that the inhibitory substance in oxLDL$_{Cu}$ is not an oxidized lipid. Studies in our laboratory indicate that the components of oxLDL that inhibit EC migration reside in the lipid fraction, and one is lysoPC.[1] With the use of thin-layer chromatography, we have shown that oxLDL$_{Cu}$ and oxLDL$_{cell}$ contain similar amounts of lysoPC (data not shown). Furthermore, α-tocopherol preserves EC migration in lysoPC.[18] Thus, differences in lysoPC content of oxLDL$_{Cu}$ and oxLDL$_{cell}$ do not explain our observations. Apparently, oxLDL$_{Cu}$ contains multiple moieties that alter membrane microviscosity, some of which are absent from oxLDL$_{cell}$, and their effects are not counteracted by α-tocopherol contained in the membrane.

Maintenance of membrane integrity by α-tocopherol could prevent activation of other systems that affect migration. Protein kinase C (PKC) activity is altered by physical properties of the membrane,[20] and α-tocopherol inhibits PKC activation by oxLDL.[15] PKC reportedly stimulates EC migration,[21] so it is unlikely to represent the mechanism by which vitamin E preserves migration. α-Tocopherol also decreases monocyte superoxide production by inhibiting the assembly of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the membrane[22] and might prevent deleterious effects of excess superoxide production by ECs in oxLDL. Further investigations are required to clarify the mechanism by which α-tocopherol preserves migration.

Observational studies have correlated increased consumption of dietary antioxidants with a reduced incidence of cardiovascular disease,[23-25] supporting the "oxidized lipid" hypothesis of atherosclerosis, but prospective randomized studies have not proven the efficacy of α-tocopherol (reviewed in Kaul et al[26] and Vivekananthan et al[27]). In a meta-analysis of seven large randomized trials of vitamin E treatment, no improvement in all-cause mortality was found in subjects taking vitamin E.[27] Most of these studies, however, focused on secondary prevention. Only two were considered primary prevention trials, but those trials enrolled older patients or patients with risk factors for atherosclerosis.[28,29] Results of the Cambridge Heart Antioxidant Study did show a benefit by a reduction in nonfatal myocardial infarctions.[25] The timing of antioxidant initiation could be important because the observational studies suggesting a striking benefit were primary prevention trials in subjects with no documented cardiovascular disease.[23,24] Furthermore, dietary antioxidants were effective in reducing transplant atherosclerosis when therapy was started within 2 years after cardiac transplantation.[30]

The mechanism of action of dietary antioxidants, including α-tocopherol, is thought to be reduction of the

oxidizability of lipids and lipoproteins, but studies to date have not documented the oxidative stress or the effectiveness of antioxidants in reducing oxidizability of lipids in a given patient. This lack of documentation is particularly important because α-tocopherol, although effective in blocking free metal ion-catalyzed oxidation reactions in vitro, does not efficiently block oxidation pathways (eg, nitration, myeloperoxidase-catalyzed chlorination, oxidative cross-linking) known to be operative in the atherosclerotic artery wall.[31-37] In a recent study, no reduction in plasma F_2-isoprostane levels (arachidonic acid oxidation products that serve as markers of global oxidative stress) was observed in subjects receiving α-tocopherol supplementation at five times normal doses (2000 IU/d × 16 weeks).[38] Those results suggest the need to critically assess current "antioxidant" regimens, monitoring oxidative stress in vivo and the ability of antioxidants to counteract the oxidative stress to rigorously test the oxidation hypothesis for atherosclerosis in humans.

Vitamin E has a number of properties in addition to its antioxidant capacity that can promote optimal cellular function. α-Tocopherol can intercalate into cell membranes and maintain membrane integrity. Our studies suggest that $oxLDL_{cell}$ increases EC membrane fluidity and inhibits migration in vitro, and the ability of α-tocopherol to preserve EC migration is dependent on the prevention of these $oxLDL_{cell}$-induced changes. A better understanding of the mechanisms involved will allow development of strategies to preserve cellular function and promote healing of endothelial injuries in vivo.

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